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Platelet Microparticles and Calcium Homeostasis in Acute Coronary Ischemias

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Elevation of free cytoplasmic calcium is the common pathway of platelet activation, leading to shape change, shedding of platelet microparticles (PMP), aggregation, and secretion of internal granules, including expression of CD62p on the surface. Platelet activation is well documented in unstable angina (UA) and acute myocardial infarction (MI). We investigated the following markers of platelet activation in 55 patients undergoing coronary angiography for suspected CAD: free cytoplasmic calcium, $[Ca^{2+}]_{cyt}$, PMP, CD62p expression, and platelet/leukocyte (P/L) interaction. $[Ca^{2+}]_{cyt}$ was measured by Fluo-3 and the other measurements were by flow cytometry. Patients were classified into three groups: unstable angina (UA, n = 11), recent myocardial infarction (MI, n = 11), and patient controls (CTL, n = 33). Blood was drawn before infusion of heparin through femoral lines at the time of catheterizaton for assays. *Results:* (1) PMP values were significantly higher in both UA and MI than in CTL, $P < 0.05$. There was no difference between UA and MI. (2) P/L interaction was significantly elevated only in UA, $P < 0.05$. (3) CD62p expression on free platelets did not differ significantly between any of the three groups. (4) The resting $[Ca^{2+}]_{cyt}$, thrombin-induced Ca^{2+} influx, and release of Ca^{2+} from internal stores were all significantly higher in platelets from the combined patient group (UA + MI) than in the patient control group, $P < 0.001$ *Conclusions:* Results on calcium hemostasis and PMP were significantly different in patients with acute coronary syndromes than those with stable angina or no coronay ischemia; this may reflect underlying pathophysiology of acute coronary ischemia. P/L interaction was higher only in the UA group, suggesting a role of leukocytes in UA. Am. J. Hematol. 54:95–101, 1997

Key words: platelet microparticles; calcium homeostasis; immune thrombocytopenic purpura (ITP); myocardial infarction; unstable angina

INTRODUCTION

There is ample evidence that coronary thrombosis occurs in patients with acute myocardial infarction (MI) and unstable angina (UA) [1], and that platelet activation plays a central role in the thrombogenesis of coronary artery occlusion [2–5]. Upon activation, platelets release microparticles (PMP) from the membrane. The role of PMP in hemostasis and thrombosis has not been fully elucidated but is believed to contribute platelet procoagulant activity by exposing anionic phospholipid surfaces for anchoring activated clotting factors in assembly of the tenase and prothrombinase complexes, accelerating clotting near the site of activation [6–9]. In Scott's syndrome, a rare congenital bleeding disorder, a defect in platelet procoagulant activity is believed to underlie the hemorrhagic tendency and has been attributed to a defect

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in PMP generation [9] and inability to expose anionic phospholipids [10]. We reported that PMP are elevated in patients with small vessel cerebrovascular accidents (CVA) [11], and in a subset of patients with acute coronary syndromes (ACS) [12], and in patients with immune thrombocytopenic purpura (ITP), particularly when thrombotic complications are present [13]. These observations suggest that PMP are a good marker of platelet activation.

In the present prospective study we investigated PMP profiles and other platelet activation markers in a series of patients with suspected coronary artery disease (CAD) undergoing cardiac catheterization for chest pain. The purpose was to determine whether elevation of PMP or other activation markers are different in patients with acute coronary ischemia, MI, or UA, as compared to control patients with stable coronary artery diseases or chest pain judged of non-cardiac origin. PMP levels were evaluated along with assays of resting Ca^{2+}_{cyt}, Ca^{2+} efflux, Ca^{2+} release from internal stores, platelet/leukocyte (P/L) interaction, and expression of P-selectin (CD62p), a granule membrane protein expressed on the surface of activated platelets as a consequence of the release reaction [14].

METHODS

Patient Selection

Patients undergoing cardiac catheterization at the University of Miami from October 1993 to May 1994 were screened for study entry. A total of 55 patients were entered into the study and gave written informed consent, approved by the IRB/Human Research Committee of the University of Miami. Prior to catheterization, a detailed history was obtained, including indications for the procedure with emphasis on the anginal pattern, presence of recent Ml, and time of the last chest pain. Medications used in the 3 days prior to catheterization were documented. Baseline data included a complete blood count (CBC), peak CPK, and MB fraction. Assays of platelet functions were performed in blind fashion, and before the classification of patients into groups was known. Patients were classified into three groups on the basis of clinical and angiographic findings as follows:

Group 1: Myocardial Infarction (MI), n = 11: Patients with documented MI by CPK or ECG changes within 6 weeks of cardiac catheterization.

Group 2: Unstable Angina (UA), n = 11: Patients with rapidly progressive chest pain and at least one episode of chest pain at rest within 5 days of catheterization.

Group 3: Patient Controls (CTL), n = 33: Consisted of (1) patients with stable angina and angiographic findings of atherosclerosis without critical lesions (n = 25); (2) patients who underwent cardiac catheterization for atypical chest pain and were found to have normal coronary arteries; (3) patients who underwent catheterizaton for evaluation of cardiomyopathy, valvular disease, or abnormal stress testing, without coronary artery disease (n=8).

Excluded from the study were (1) patients with recent history of non-cardiac thrombosis (pulmonary embolism, deep venous thrombosis [DVT]), CVA, and transient ischemic attacks (TIA); (2) patients with systemic disorders or inflammation associated with coagulopathy, hypercoagulable states, or abnormal platelet count (low or high); (3) recent severe bleeding or surgery within 6 weeks. Not excluded were patients with diabetes mellitus, hypertension, hyperlipidemia, and peripheral vascular disease.

Blood Sampling

A standard arterial sheath was inserted into a femoral or brachial artery for cardiac catheterization. All sheaths were 5 French or larger and 12 cm long. The sheath was flushed with 5 mL heparin flush (1,000 U heparin/100 mL NS). Blood (30 mL) was withdrawn and used for other studies, then a 30 mL syringe containing 3 mL anticoagulant citrate dextrose (ACD) was attached to the arterial sheath port and blood was allowed to enter the syringe passively to a volume of 30 mL.

Assay of Platelet Microparticles (PMP)

The method used has been described elsewhere [15]. Briefly, platelet-rich plasma (PRP) was prepared by centrifuging whole blood 10 min at 160g; platelet-poor plasma (PPP) was prepared by centrifuging PRP for 6 min at 2,000g. To 20 μL of PPP was added 5 μL of FlTC-conjugated α-CD41 or PE-conjugated α-CD42 (both from Coulter/Immunotech, Inc., Miami, FL). After 20 min, 25 μL of 4% p-formaldehyde (PFA) was added, and then after at least 20 min fixation, 2 mL of PBS was added and the tubes were vortexed. PMP were measured in the Profile II flow cytometer (Coulter Corp., Miami, FL) with neutral density filter removed and triggering on fluorescence. Results are expressed as PMP $\times 10^7$/mL.

Assay of Activation Marker CD62 [16]

Surface expression of the platelet alpha granule protein CD62p (a.k.a. PADGEM, or GMP-140, now P-selectin) was determined flow cytometrically by measuring bound PE-conjugated α-CD62p (AC1.2, Becton-Dickinson, San Jose, CA). To 10 μL PRP was added 4 μL a-CD62p, then after 10 min, 15 μL 4% PFA, then after 15 min, 1 mL PBS. Events were recorded by light-scatter triggering. Results are expressed as total fluorescent intensity, i.e., product of number of CD62+ events by mean fluorescent intensity.

Assay of Platelet-Leukocyte (P/L) Interaction

To 50 μL whole blood was added 50 μL PBS, 4 μL of FlTC α-CD41, and 4 μL PE α-CD45 (Sigma, St. Louis, MO; #p-7687). After 10 min incubation, 100 μL 4% PFA was added, then 15 min later, 1.0 mL PBS was added and the sample was ready for flow cytometry. The flow rate was 20 μL/min and counting time was 60 sec; therefore the effective volume of original blood measured was 1 μL. The instrument was set to trigger on red fluorescence from PE-labeled pan-leukocyte marker, α-CD45. By criteria of side- and forward-scattered light, a bitmap was drawn around the neutrophil population, identified as such by previous experiments with isolated neutrophils and confirmed by labeling with α-CD11b (Coulter/Immunotech, Miami, FL). This population was analyzed on a separate histogram whose x-axis was green fluorescence (from FITC-labeled α-CD41) and whose y-axis was red fluorescent intensity. Coexpression of both was taken to indicate association of platelets with leukocytes (neutrophils). It has been shown by Rinder et al. [17] that platelets interact almost exclusively with neutrophils and monocytes, not lymphocytes. Results are expressed as total fluorescent intensity, as above.

Intracellular Free Calcium $[Ca^{2+}]_{cyt}$ and Releasability of Ca From Internal Stores

This was measured in 13 patients (6 UA, 7 MI) and 17 patient controls (stable pain) by the method of Fluo-3/AM (Molecular Probes, Inc., Eugene, OR; catalogue no. F1242) [18]. Platelets in PRP were loaded with Fluo-3 (10 μM) for 30 min and the excess removed by centrifuging twice and resuspending in Ca^{2+}-free Tyrode's buffer. Then 2 mL of the cell suspension at 1×10^7/mL was added to an acrylic cuvette clear on all sides and Ca^{2+} was measured on a Perkin-Elmer Spectrofluorometer Model LS 50B, using ex = 506 nm and em = 526 nm. Four measurements of $[Ca^{2+}]$cyt were made on each sample, (1) initial resting; (2) peak value after thrombin stimulation in the presence of external 2 mM Ca^{2+}; (3) peak value after thrombin stimulation in the presence of external 2 mM EDTA (the latter to measure $[Ca^{2+}]_{cyt}$ released from internal stores only); (4) peak value following treatment with ionomycin (Calbiochem Inc., La Jolla, CA) in presence of EDTA external.

Statistical Analysis

To determine baseline differences between groups, Fisher's exact test was used for categorical variables and the unpaired Student's *t*-test was used for continuous variables. Normality was assessed with the Kolmogorov-Smirnov test. Analysis of variance and Scheffe's procedure were used to determine differences of PMP values between groups.

RESULTS

Baseline data on the three groups (age, sex, blood counts, risk factors, medications) are compared in Table I.

PMP Assay Results

Mean PMP levels as measured by CD41 expression (GP IIb/IIIa) were nearly twice as high in the recent MI group and were also significantly elevated in unstable angina (UA) compared to patient controls (CTL), $P < 0.05$ by ANOVA (see Fig. 1). There was no significant difference between the UA and MI groups (group 2 vs. group 3).

Calcium Studies

We investigated the sensitivity of platelets from MI and UA patients to thrombin-induced Ca^{2+} influx and release of Ca^{2+} from internal stores, and the effect of the Ca^{2+} ionophore, ionomycin. Figure 2 shows the results of these studies. The two patient groups, UA and MI, were combined for this purpose. Error bars show standard deviation (SD). Notice in Figure 2A that the resting (unstimulated) $[Ca^{2+}]_{cyt}$ was elevated in UA/MI compared to patient controls (177 ± 51 nmol/L in UA/MI vs. 120 ± 37 in CTL; $P < 0.001$). Following addition of thrombin (0.1 U/mL) in the presence of external Ca^{2+} (2 mmol/L), the UA/MI group showed much greater response (651 ± 210 nmol/L in UA/MI vs. 400 ± 120 in CTL; $P < 0.001$) (see Fig. 2B). When thrombin was added in the absence of external Ca^{2+} (presence of EDTA 1 mmol/L), the UA/MI group released more Ca^{2+} from internal stores compared to patient controls (455 ± 113 nmol/L in UA/MI vs. 270 ± 81 in CTL; $P < 0.001$) (Fig. 2C). To estimate the total releasable Ca^{2+} in the platelet organelles of UA/MI vs. patient controls, ionomycin (5 μmol/L) was added to allow complete efflux of Ca^{2+} from the organelles to the cytosol and to the external medium (EDTA present): as seen in Figure 2D, the UA/MI group contained almost 2-fold more organelle-sequestered Ca^{2+} (1148 ± 433 nmol/L in UA/MI vs. 642 ± 330 in CTL; $P < 0.05$).

Platelet Activation Marker CD62

Results on expression of activation marker CD62 is shown in Figure 3. Mean values are slightly elevated in MI and are about doubled in UA as compared to controls (CTLs). However, these results did not achieve significance in this small patient sample.

Platelet/Leukocyte Interaction

Leukocytes that were positive for FITC-labeled platelets were also quantitated [see 20]. As shown in Figure 4, this measure was significantly elevated only in the UA group ($P < 0.05$ by ANOVA), not in the MI group.

Comparison of baseline data of Table I shows that the

TABLE I. Baseline Parameters of Patient and Control Groups*

	I. Recent MI (n = 11)	II. Unstable angina (n = 11)	III. Patient control (n = 33)	Recent MI vs. CTL (*P* value)	Unstable angina vs. control (*P* value)
Age	54.4 ± 11.4	53.7 ± 8.7	58.3 ± 12.6	ns	ns
Percent males	90.9	90.9	87.9	ns	ns
Platelets	276 ± 178.0	243.8 ± 63.7	223 ± 68.9	ns	ns
WBC	9.8 ± 2.3	9.4 ± 5.1	7.7 ± 2.0	0.0081	ns
Hematocrit	40.4 ± 6.3	42.8 ± 4.7	41.7 ± 4.9	ns	ns
Risk factors					
Sum of risks factors	3.1 ± 1.4	3.9 ± 1.0	2.5 ± 1.1	ns	0.0032
Hypertension (%)	63.6	80.0	59.4	ns	ns
Diabetes mellitus (%)	45.5	40.0	25.0	ns	ns
Hypercholesterol (%)	36.4	60.0	18.8	ns	0.0200
Family history (%)	27.3	50.0	18.8	ns	ns
Smoking (%)	63.6	70.0	45.5	ns	ns
Medications					
β blocker (%)	72.7	54.5	30.3	0.0031	ns
Calcium blocker (%)	9.1	90.9	46.9	0.0330	0.0140
ACE (%)	18.2	18.2	34.4	ns	ns
Nitrates (%)	90.9	81.8	43.8	0.0120	0.0290
Aspirin (%)	100.0	72.7	65.6	0.0410	ns

*Variables are expressed as the mean ± standard deviation. Significance were determined by Student's *t*-test for continuous variables and Fisher's exact test for categorical variables; ns = no significant difference.

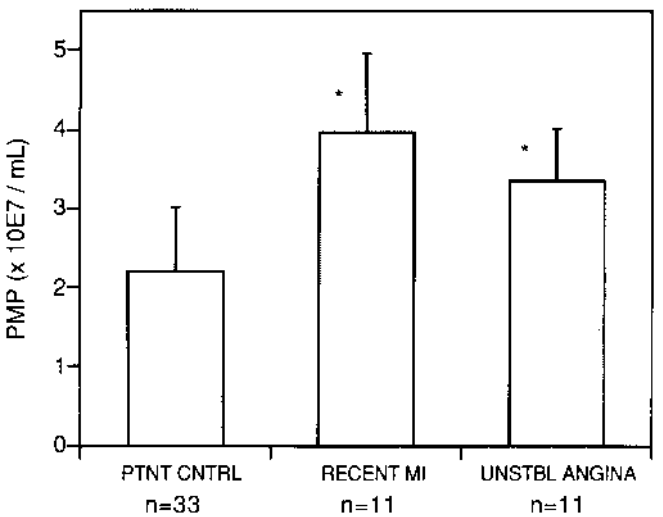

Fig. 1. Mean PMP levels in the patient and control groups. Eror bars show 1 SD. Star (*) indicates $P < 0.05$ relative to patient controls. The difference between recent MI and unstable angina groups was not statistically significant.

three groups were well matched for age, gender, hematocrit, diabetes, hypertension, smoking, and family history. Hypercholesterolemia was significantly higher in the UA group and white cell counts were higher in the MI group. Statistical analysis of medications showed that calcium channel blockers and nitrates were used more frequently in ischemia groups (MI and UA). Both are known to inhibit platelet activation, and therefore would depress PMP and $[Ca^{2+}]_{cyt}$ values; but both groups had elevated levels in both assays, suggesting that in the absence of these medications, differences would be larger than observed. Beta blocker and aspirin were more frequently used in MI group. Both inhibit platelet activation, therefore activation assays could be higher than observed if the medication was absent.

DISCUSSION

Platelet activation is initiated by the binding of an agonist to its corresponding receptor on the platelet surface, resulting in activation of a common pathway producing a transient rise in intracellular calcium, followed by shape change, membrane alteration, exposure of additional antigens and receptors, shedding of microparticles (PMP), adhesion, aggregation, and secretion of internal granules [19,20]. Of the many assays employed to detect platelet activation in thrombotic conditions, assay of platelet calcium handling is of particular interest since Ca^{2+} is the common intracellular messenger of cell activation: elevation of $[Ca^{2+}]_{cyt}$ is a necessary and sufficient event for platelet activation [21], i.e., for shape change, aggregation, and secretion. Platelet activation has in turn been shown to be a major factor in initiation of arterial thrombosis [22], leading to UA and/or MI under certain conditions [23].

We have previously shown that both resting $[Ca^{2+}]_{cyt}$ and dense tubule-sequestered total Ca^{2+} are elevated in patients with thrombosis, including UA and MI [24]. The present study substantiates that report and leads to the further inference that the principal difference in platelet Ca^{2+} handling in UA/MI vs. patient controls consists of greater Ca^{2+} releasability from internal stores, rather than

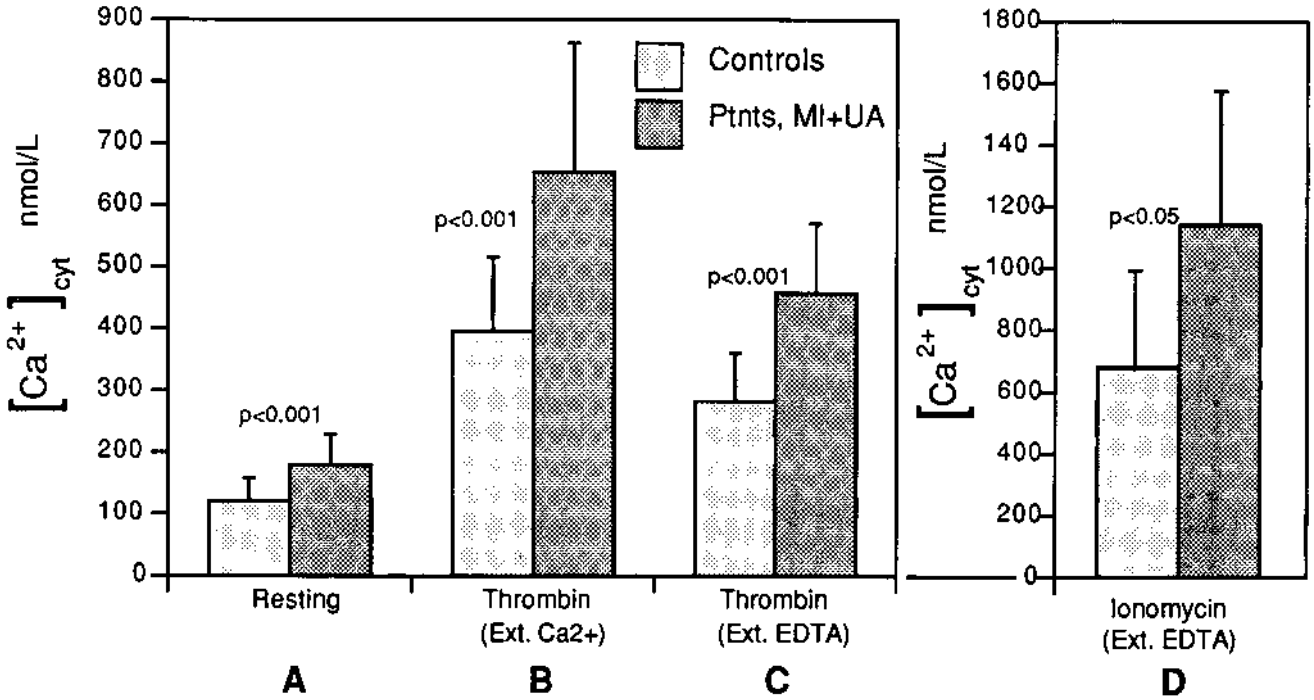

Fig. 2. Summary of calcium homeostasis experiments. A: initial resting $[Ca^{2+}]_{cyt}$; B: peak value after thrombin stimulation in the presence of external 2 mM Ca^{2+}; C: peak value after thrombin stimulation in the presence of external 2 mM EDTA (to measure Ca^{2+} released into the cytoplasm from internal stores only); D: peak value following treatment with ionomycin in presence of external EDTA. The two patient groups, UA and MI, were combined for these studies, n = 13 patients (UA + MI); n = 17 patient controls. Error bars show standard deviation (SD).

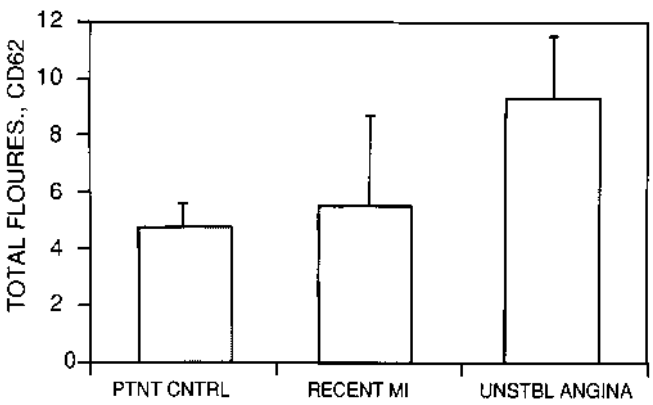

Fig. 3. Expression of activation marker, P-selectin (CD62p). Total fluoresence is defined as the product of mean red flourescent intensity (due to PE label on α-CD62p) by the number of CD62+ platelets counted. Numerical results were divided by 100 to give the scale of the y-axis. Error bars show standard error of the mean (SEM). The MI group did not differ significantly from controls.

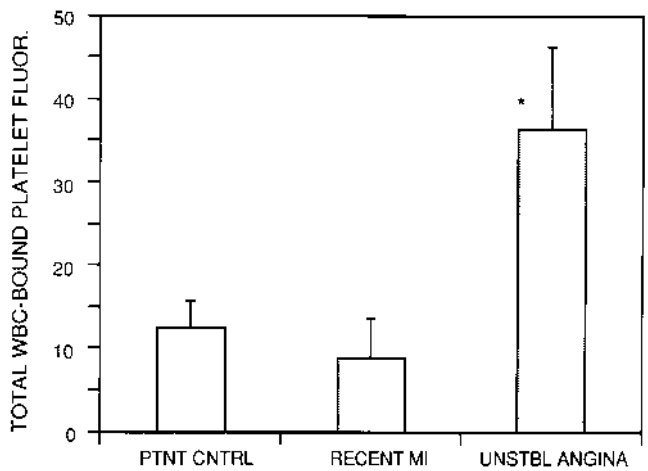

Fig. 4. Platelet-leukocyte association (interaction). The value given on the y-axis of the figure is the product of the mean fluorescent intensity of FITC (platelets) by the number of CD41+ leukocytes. It is seen that the myocardial infarct (MI) group does not differ from patient controls in this measure, but unstable angina (UA) is sharply elevated, $P < 0.05$.

greater Ca^{2+} influx (because the difference between UA/MI and patient controls in Fig. 2C, representing release from internal stores only, is 185 nmol/L, or about 75% of the difference between UA/MI and patient controls seen in Fig. 2B, the latter representing the sum of Ca^{2+} release from internal stores and influx through the plasma membrane). This result may be a consequence of the greater total releasable Ca^{2+} found in platelets of UA and MI patients implied by Figure 2D and as earlier reported

[24]. The accumulation of more Ca^{2+} in the platelets of UA/MI as compared to CTL could be a consequence of the greater Ca^{2+} permeability of the plasma membrane of partially activated platelets, resulting in greater uptake into the internal organelles, as previously suggested [25]. The platelet calcium levels reported here may not reflect the true values in vivo, since handling involved in the assay may introduce artifacts. However, the relative differences observed between patient and control groups are not readily explainable as artifacts, and indicate that the assay distinguishes abnormal calcium handling in platelets of ischemic groups.

In the present study assay results of PMP, platelet Ca^{2+}, CD62 expression, and P/L interaction in patients with UA or MI were compared to those wih stable anginal syndromes and non-coronary disorders. Results on $[Ca^{2+}]_{cyt}$ exhibited the largest difference between the UA or MI groups and those with stable conditions. However, assay of $[Ca^{2+}]_{cyt}$ by fluorescent probes is tedious and requires special expertise, while PMP assay is simple, economical, and rapid, and is therefore better suited as a clinical test for screening/monitoring. Interestingly, assay of P/L interaction discriminated UA from MI (Fig. 3), suggesting that leukocytes are more involved in the thrombogenesis of UA than acute MI.

Also unexpected is that CD62 expression in platelets is not significantly elevated in the recent ischemic group, even though both PMP and Ca^{2+} is. This may reflect variations in the steps or pathways of platelet activation. For example, platelet activation may not necessarily proceed to granule secretion and P-selectin expression (CD62+); alternatively, CD62+ platelets may be immediately recruited to P/L aggregates, escaping detection of CD62 in free platelets. We recently described such a phenomenon in a study of patients with thrombotic thrombocytopenic purpura [26].

Tate et al. found increased PMP in patients with unstable coronary syndromes [27]. However, they did not differentiate UA patients from those with acute MI, and their samples were drawn from the coronary sinus, thus leaving unanswered whether there is systemic evidence of platelet activation as measured by PMP levels. The present study confirms that there is systemic activation as measured by PMP and by $[Ca^{2+}]_{cyt}$. Measurement of platelet Ca^{2+} in the limited number of patients in this study showed significant elevation in all four parameters (Fig. 2A–D) in patients with recent ischemias as compared to patient controls. This indicates pre-activation of platelets in patients with acute coronary syndromes, rendering them hypersensitive to agonists. These observations are consistent with the elevated PMP concentration, and with the well-known increased risk of a second cardiac event.

It is possible that differences in medications in the three groups (Table I) could have affected results. The MI and UA groups received more antiplatelet drugs (which tend to depress activation) than controls, yet show higher activation. However, the possibility that medications influenced the higher P/L interaction in the UA group compared to the MI group cannot be ruled out. A larger-scale study would be needed to investigate the effect of medications on assays of platelet activation.

In summary, PMP and $[Ca^{2+}]_{cyt}$ appear significantly elevated in UA and recent MI. Although $[Ca^{2+}]_{cyt}$ assay is more sensitive, PMP assay is more rapid and economical. Platelet-leukocyte (P/L) interaction was elevated only in the UA group, not in MI. Further study is needed to refine these methods for possible clinical applications, and to better evaluate the role of P/L interaction in thrombotic conditions.

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